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OSTEOARTHRITIS and CARTILAGE

Transplantation of allograft chondrocytes embedded in agarose gel into cartilage defects of rabbits

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Summary

Objective: Durable healing of full-thickness articular cartilage defects has been considered for a long time as a highly desirable, but unlikely event to occur. In recent years, conflicting reports on the outcome of *in vitro* and *in vivo* studies on chondrocyte and cartilage grafting into animal and human joints have raised new arguments for and against controlled repair of articular cartilage following injury. Some of the problems result from insufficient characterization of implant and repair tissue, and from too short follow up phases. Here we describe a new approach to repair articular cartilage defects in rabbit knees by allografting chondrocytes cultured in agarose gels.

Design: The implants were monitored over 6–18 months and graded histologically, immunohistochemically, and electron microscopically, using a grading scale based on seven evaluation criteria. Control implants of pure agarose produced poor fibrous substitute tissue, insufficient healing and incomplete filling of the cartilage defects. After transplantation of allogenic chondrocytes embedded in agarose, the quality of the newly formed repair cartilage was superior with respect to type II collagen and proteoglycan content and cellular architecture when compared with untreated defects. Superficial fibrillation and degradation were significantly diminished or prevented.

Results: New subchondral bone formed at the level of the previous subchondral bone. In most cases the repair tissue merged with the host articular cartilage; normal calcified cartilage was the only tissue zone that did not participate in the integration of the transplant. By gross evaluation 24% of grafts showed an extent of recovery never observed in controls. The best results were obtained after 18 months when 47% of the grafts ($N = 15$) developed a morphologically stable hyaline cartilage.

Conclusion: These studies demonstrate that agarose-embedded chondrocyte may prove a valuable tool for controlled repair of articular cartilage defects.

Key words: Agarose gel, Articular cartilage, Chondrocytes, Transplantation.

Introduction

Adult articular cartilage has got a limited ability to regenerate defects caused by injuries or degenerative events. During repair attempts of full-thickness defects, mesenchymal cells migrate into damaged areas and reorganize a cartilaginous tissue, but this is generally not a functionally intact hyaline cartilage [1]. Rather, the formation of a fibrocartilaginous tissue is initiated which is unstable and destined for ultimate degeneration [2], often progressing to osteoarthrosis. In partial thickness defects cartilage regeneration is even more difficult and rare [3, 4].

Different methods were developed to transplant osteochondral allografts, periosteal grafts and isolated chondrocytes into full-thickness defects in order to facilitate the repair of articular cartilage [5–7]. The reconstruction of cartilage defects using osteochondral allografts has been proven to be a suitable treatment for traumatic lesions only, but after longer periods the viability of the allograft decreased [8, 9]. Moreover, the immune response to the osteochondral allografts presented an unsolved problem [10]. The use of periosteal autografts with their osteogenic and chondrogenic potential enhanced the formation of hyaline cartilage in comparison to natural healing [5], particularly if combined with TGF-β [11] or with the injection of precultivated autologous chondro-

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cytes [6, 12, 13]. Transplantation of chondrocytes or chondrogenic cells seemed to play a specific role, since they are able to regenerate a functional and biomechanically intact extracellular matrix and to preserve its functional stability for a longer period. Transplantation of chondrocytes or chondrogenic cells alone was shown to be successful in rabbit models, but the healing rate was limited due to loss of viability in the transplanted cells and due to the difficulty of fixing chondrocytes in the defect [6, 8, 14, 15]. Brittberg *et al*. [12] reported positive, convincing repair results after treatment of deep cartilage defects in the knees of patients up to 66 months after operation. Cultured autologous chondrocytes were injected under a periosteal flap which was sutured over the defect.

A series of studies made evident that the culture conditions used for cultivation of chondrocytes were critical for the maintenance of the cartilaginous phenotype including its characteristic metabolic activities. Embedding chondrocytes into gels is advantageous in comparison to monolayer cultures [16, 17]. During serial monolayer cultures chondrocytes stop producing cartilage specific proteoglycans and type II collagen, and switch to type I collagen and produce lower levels of proteoglycan [18–21]. Such dedifferentiated chondrocytes re-express their original phenotype during culture in agarose gel [16, 22]. But obvious difficulties were seen in the use of complete agarose gels containing chondrocytes in grafting experiments because of its consistency and because of possible immunological reactions against these matrix molecules. Besides biodegradable polymers [23, 24], collagen gels were preferred as vehicles in culturing chondrocytes in three dimensional systems before transplantation [25, 26]. In most studies type I collagen isolated from the skin was used. It appears less suitable in reconstituting hyaline cartilage as it is absent from normal articular cartilage. Cultivation of chondrocytes in collagen gel was described previously, where they maintained their phenotypical properties [27]. However, it was also shown, that culturing of human chondrocytes in a collagen gel induced dedifferentiation [28] and loss of pericellular proteoglycans and type II collagen [29].

Therefore it is the aim of this study to investigate the repair of full-thickness defects of

Table I
Selected criteria for the evaluation of repaired areas

Criterion Valuation	Criterion Valuation
Proteoglycan staining	Type II collagen staining
0 Without proteoglycan	0 Without type II collagen
0.25 Weak, about 25%/only residual	0.25 Weak, about 25%/only residual
0.5 about 50%/irregularly distributed	0.5 about 50%/irregularly distributed
0.75 about 75%	0.75 about 75%
1.0 Normal	1.0 Normal
Character of the superficial zone	Arrangement of chondrocytic cells
0 Strong fibrous and type II collagen negative layer with extensive splitting and destruction	0 Very heterogenous/only fibroblast like cells
0.25 Strong fibrous and type II collagen negative layer/split at the surface	0.25 Hypocellular/heterogenous distribution and arrangement
0.5 Somewhat fibrous and type II collagen negative layer, no surface fissures	0.5 Preferentially clusters of cells/even distribution
0.75 Reduced in type II collagen, but not fibrous	0.75 Columnar structure in parts
1.0 Normal, like superficial cartilage zone	1.0 Normal, like in zones of articular cartilage
Ossification/border between repair cartilage and subchondral bone	Merging of repair cartilage with adjacent residual cartilage
0 Complete ossification of repair tissue	0 Not existing
0.25 Repair tissue influenced by extensive ossification	0.25 Only poorly existing/disturbed by shifted layers
0.5 Beginning to narrow the cartilage	0.5 Impaired, inhomogenous
0.75 Still showing contours of the original defect	0.75 Only with upper layer above tidemark
1.0 Resembling the adjacent normal area	1.0 Nearly without disturbances (normal)
Tidemark in the repaired area	
0 Not existing	
0.25 Only partly existing/very heterogenous	
0.5 Incomplete	
0.75 Impaired	
1.0 Complete, normal	

rabbit knees by allograft chondrocytes embedded in agarose. The chondrocytes were precultivated in an agarose gel until production of extracellular matrix halos was visible around the cells. The production of a pericellular cartilage matrix provided an appropriate environment for a stable chondrocyte phenotype after implantation and the correct scaffold for hyaline cartilage matrix deposition. In view of the instability of the chondrocyte implants and alterations of the repair tissue reported in previous studies [1], we decided to perform long-term studies up to 18 months.

The joints were evaluated with conventional histochemistry of proteoglycans and immunostaining of type II collagen after 6–18 months. The grafting experiments were compared with spontanous healing and with transplants of pure agarose.

Materials and Methods

ISOLATION AND CULTIVATION OF CHONDROCYTES

Articular cartilage prepared from femoral heads of 6–8 week-old rabbits (Chinchilla bastard) was cut in small pieces. The chondrocytes were liberated by sequential digestion with pronase E from *Streptomyces griseus* (Serva 33635) (2 mg/g cartilage, 30 min, 37°C) and with collagenase from *Clostridium histolyticum* (Serva 17449) (1 mg/g cartilage, 8 h, 37°C). Liberated cells were washed and resuspended in Ham's F12 medium. The isolated chondrocytes were cultured in agarose as described [16, 22]. Briefly, 40-mm Petri dishes were coated with a thin layer of 1% agar (Difco, Detroit, U.S.A.). Then 2% agarose of high electroendosmosis (Serva 11397) was equilibrated to 37°C after autoclaving and an equal volume of Ham's F12 medium containing 100 units/ml

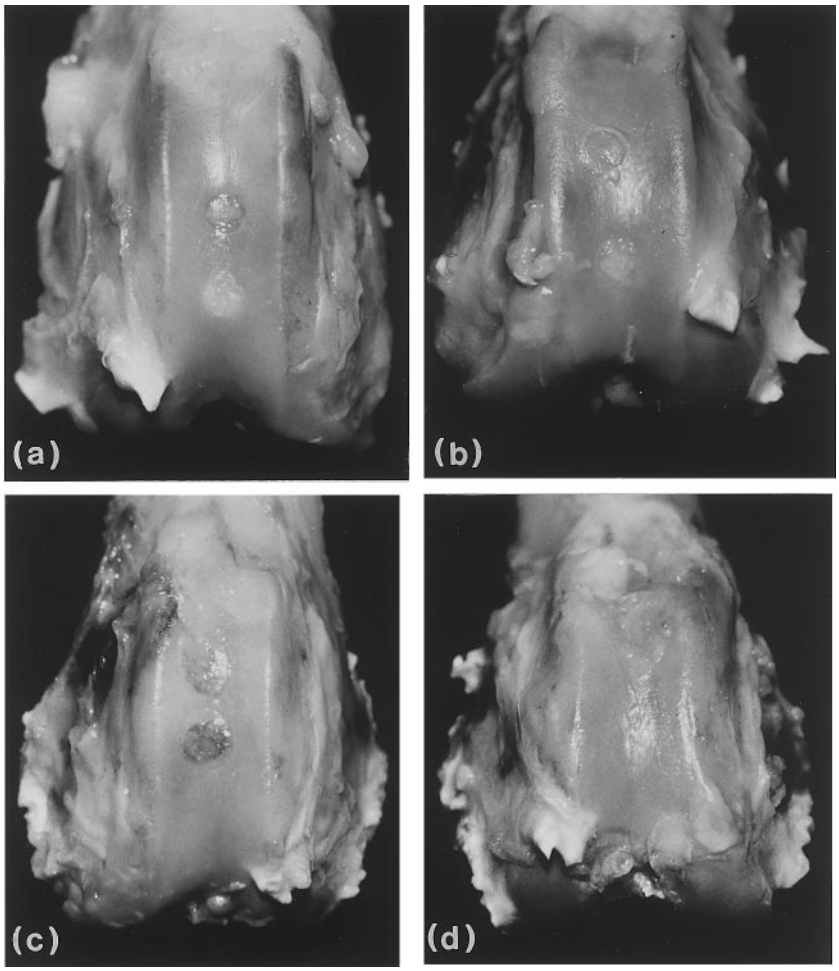

FIG. 1. Macroscopic appearance of defects in patellar grooves; (a) control defects 9 months after surgery, (b) chondrocyte agarose transplanted defects 9 months after surgery, (c) control defects 18 months after surgery, (d) chondrocyte agarose transplanted defects 18 months after surgery.

penicillin and 100 μg/ml streptomycin was added. This solution was mixed with one volume part of cell suspension to yield a final density of 1.5×10^6 cells/ml and added to the precoated Petri dishes. After gelation at 4°C for 10 min the embedded cells were overlayed with Ham's F12 medium supplemented with antibiotics and 1 mM cysteine [30]. The cells grew to a density of about 2.5×10^6 cells/ml for an additional 10–14 days in a humidified atmosphere with 5% CO_2. Every two days the medium was renewed by the above-mentioned medium containing 2% fetal calfserum.

TRANSPLANTATION OF CHONDROCYTES

Animals were anesthetized with 4 ml of a mixture of 0.5% xylazine, 7.5% ketamine i.m.; if necessary 0.5 ml 0.2% xylazine, 3% ketamine in physiological salt were added i.v. Full-thickness articular surface defects of 3 mm diameter completely penetrating the entire cartilage into the subchondral bone (1.5 mm depth) were drilled in the medial and lateral femoral condyles and two defects in the intercondylar grooves of adult female Chinchilla bastard rabbits (nine animals). The area was moistened with antibiotics (Nebacetin®, Byk Gulden, Konstanz, FRG), a drop of a fibrin adhesive system (Tissucol-kit®, Immuno, Heidelberg, FRG) was applied into the defects, which were immediately filled with the chondrocyte-agarose gel. For this purpose, a small piece of the culture layer of the gel was cut off some distance from the margin and grafted onto the defect with the upper side on the surface. Excess material was removed carefully. In the control group, the defects were left empty and in a third group they were filled by agarose gel without cells. After clot formation the arthrotomy was closed surgically. Sixteen chondrocyte grafts, six empty control defects and two defects filled with agarose without cells were obtained at each experimental time point of 6, 12, and 18 months, respectively.

PREPARATION OF MATERIAL FOR HISTOLOGICAL EXAMINATION

The rabbits were killed after the indicated time periods and then the femurs were prepared. The femur heads were sawn to pieces containing the individual defective areas. The pieces were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2 days, hydrated for 1 day, decalcified in 10% ethylenediaminetetraacetic acid (EDTA) in PBS for at least 2 weeks, dehydrated and embedded in paraplast according to routine methods.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STAINING

Serial sections of 8 μm thickness were cut from the embedded tissue specimens perpendicular to the articular surface and mounted onto microscopic slides (Superfrost Plus, G. Menzel, Braunschweig, FRG). Proteoglycan staining was performed by toluidine blue and safranin-O [31]. For immunochemical detection of type II collagen a monoclonal mouse antibody against type II collagen from chicken (CII E8) was used [32]. A

FIG. 2. Photomicrographs of control defects 12 months after surgery; immunohistochemical staining of type II collagen; surveys (original magnification 25×). (a) In a medial condyle fibrous repair tissue filled the defect with complete apposition to the residual cartilage only at the left side. Superficial degradation and ossification protruding from the subchondral bone contribute to the loss of cartilage (arrow). (b) In a patellar groove the defect was not filled with replacement tissue and opened to a bone cavity filling it with fibrous tissue.

FIG. 3. Defect in a lateral condyle filled with pure agarose, 14 months after surgery; toluidine blue staining; (a) survey (original magnification 25×), (b) detail (original maginification 100×). In the centre of the defect connective tissue like material was excessively formed and apparantly had its source in the bone marrow.

FIG. 4. Chondrocyte agarose-filled defects in a medial condyle 6 months after transplantation (a) and in a lateral condyle 12 months after transplantation (b) in details (original magnification 100×). In (a) there is no connection between implant and host cartilage stained for type II collagen. (b) The original transplant tissue (left) was partly substituted by ossification, the remaining part merged with the host cartilage above the tidemark. The surface showed fibrous degeneration and fibrillation. (Polarized light microscopy combined with immunohistochemical staining for type II collagen).

FIG. 5. Chondrocyte agarose-filled defect in a medial condyle 6 months after transplantation; toluidine blue staining; (a) survey (original magnification 25×), (b) detail (original magnification 100×). The defect was completely filled with cartilaginous tissue.The cells within the defective area were arranged heterogenously; chondrocyte clusters and columnar cartilage-like structures were visible in the depth and with a tangential zone-like layer at the surface. The gaps between repair and original cartilage might be caused through histological procedures, nevertheless they demonstrate failure of merging with the surrounding host tissue in this case.

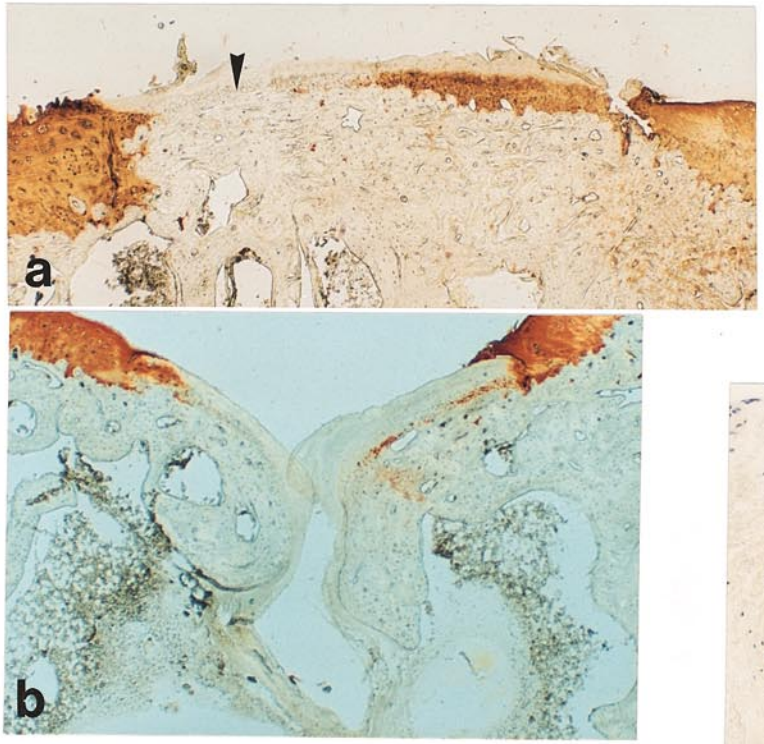

Fig. 2

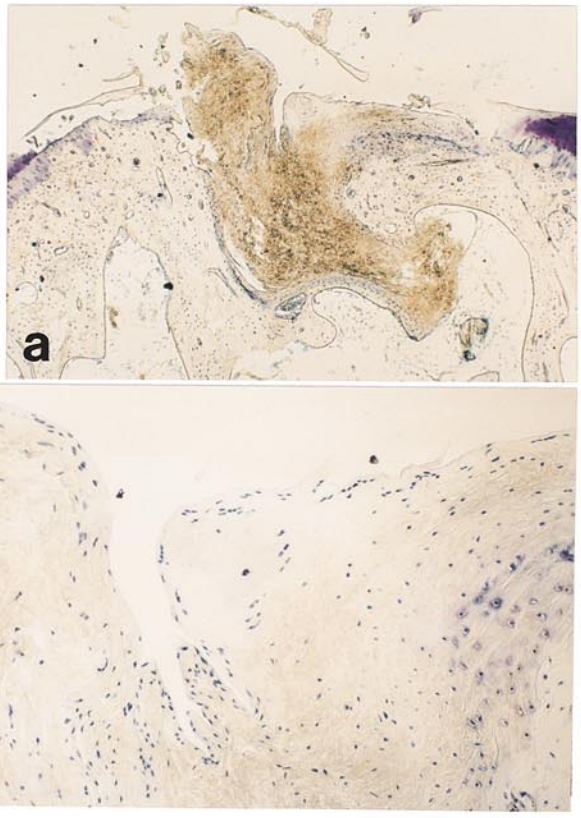

Fig. 3

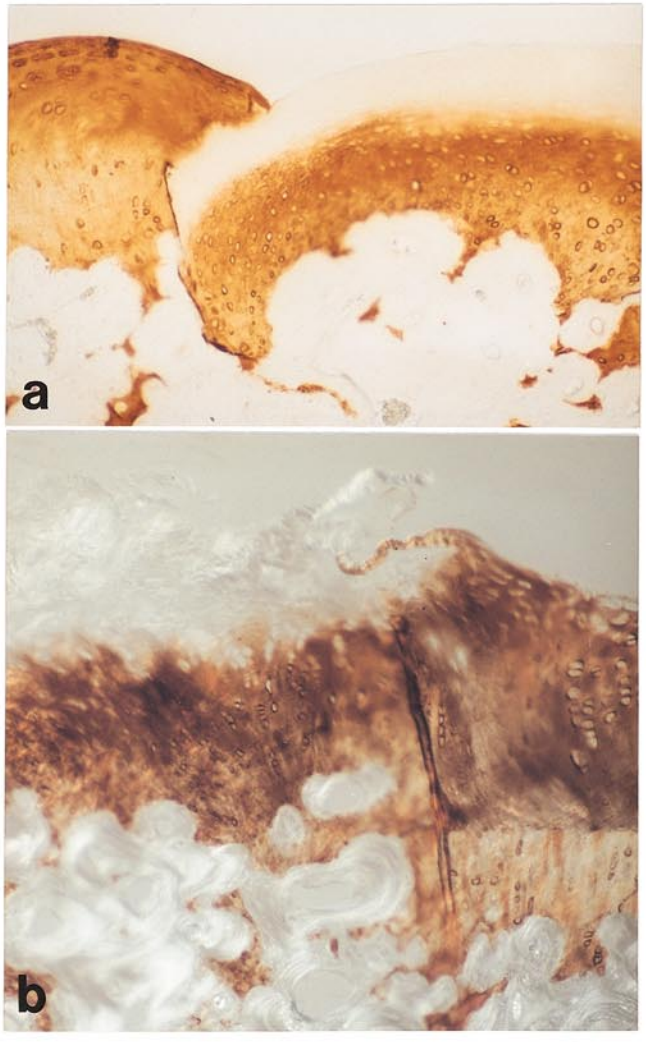

Fig. 4

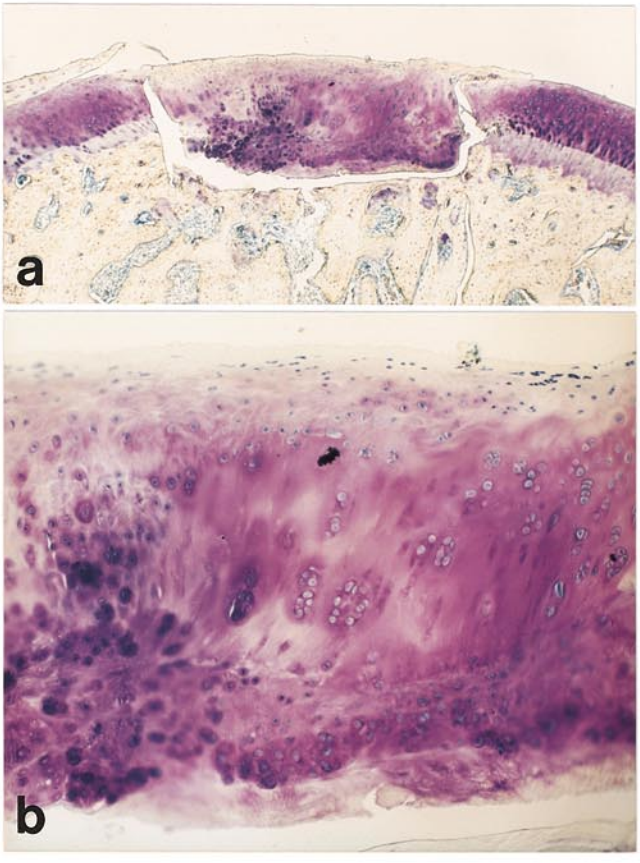

Fig. 5

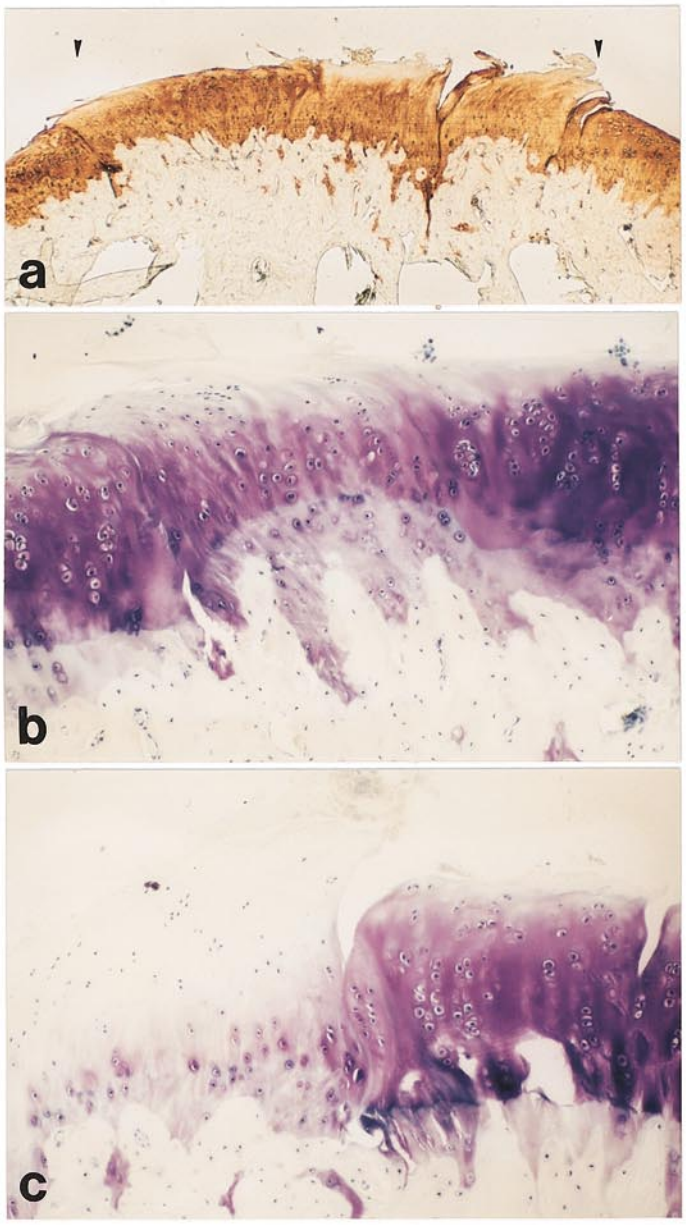

Fig. 6

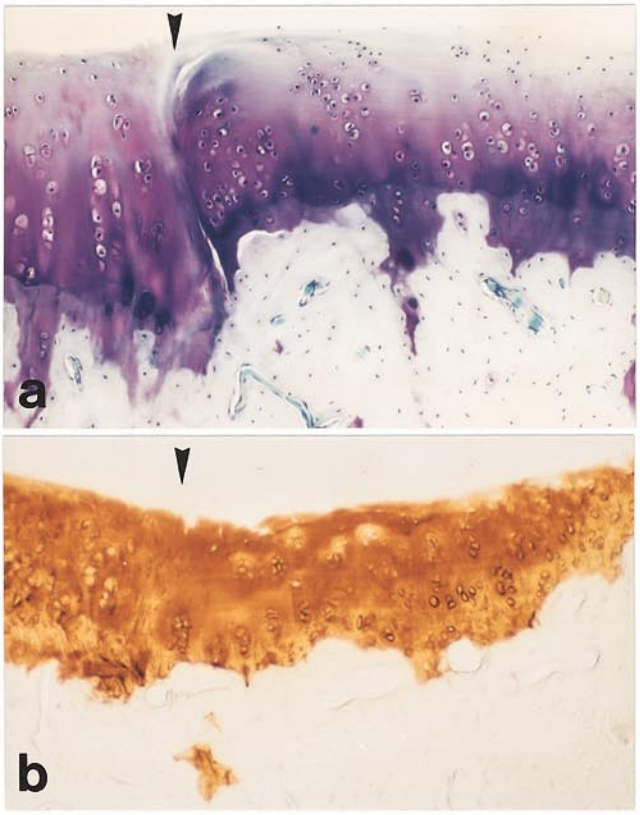

Fig. 7

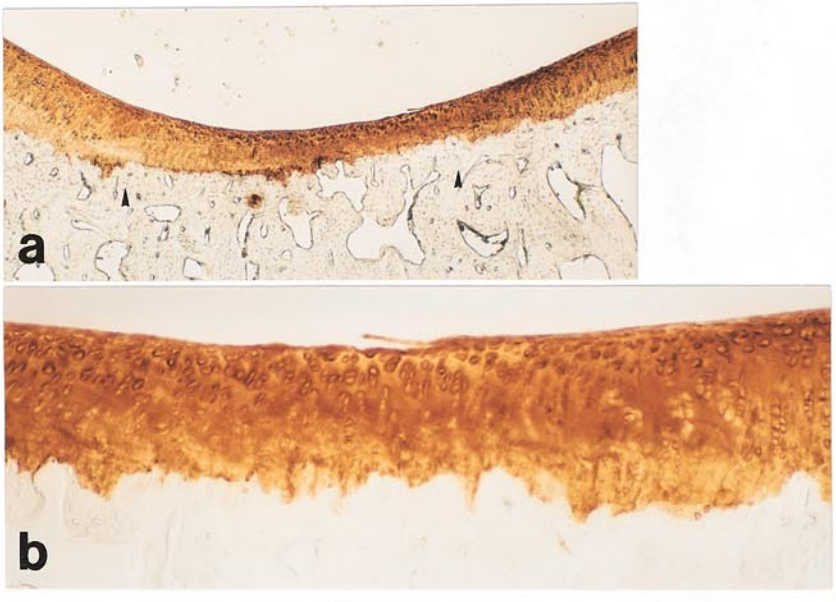

Fig. 8

peroxidase-conjugated goat anti-mouse IgG (A-2554 from Sigma, Deisenhofen, FRG) served as secondary antibody. Briefly, deparaffination of the sections was followed by subsequent incubation steps: 5 min PBS; 30 min 50 mM EDTA pH 7.5 in PBS; 40 min 0.7 mg/ml (2500 IU/ml) hyaluronidase (Sigma H6254) in PBS at 37°C; 3×5 min PBS; 30 min 1 mg/ml (about 1000 PUC-U/mg) pronase E from *Streptomyces griseus* (Serva 33635) in 50 mM Tris/HCl pH 7.6 at 37°C; 3×5 min PBS; 30 min blocking reagent (Boehringer Mannheim, FRG) with 0.3% Tween 20; 14 h primary antibody (undiluted culture supernatant CII E8); 2×5 min PBS (including several changes of PBS at the beginning, applicated in drops); 1 h secondary antibody (1:200 diluted in 1/10 blocking reagent with 0.03% Tween 20); 2×5 min PBS (including several changes of PBS at the beginning); 10–30 min substrate solution consisting of 0.067% (w/v) 3,3'-diaminobenzidine tetrahydrochloride and 0.024% hydrogen peroxide in Tris buffered saline (50 mM Tris/HCl pH 7.6, 150 mM NaCl); 5 min PBS. Finally, the specimens were mounted in glycerol gelatin (Sigma). The procedure was checked for sufficient cross reactivity of the primary antibody and specificity of rabbit type II collagen detection with the aid of tissue sections of fetal rabbit joints as substrate.

ANALYSIS OF HISTOLOGICAL PREPARATIONS

Sections of all 72 defects (48 grafts, 18 controls, 6 filled with agarose) were examined. Half of them presented condylar defects, the other half descended from patellar grooves in each group. According to the procedure of O'Driscoll, Keeley and Salter [5] all specimens were evaluated in detail by seven different restitution parameters (Table I). In order to determine correlations between the selected criteria, the time course and the different experimental groups, each specimen was scored on a scale of 0 to 1 (0–100%) for each parameter (Table I). For overall assessment, single scores were added to obtain a total point value for each defect. Theoretically, perfect healing of a defect was characterized by 7.0 points as seven parameters were evaluated. Means and standard deviations of each criterion were estimated within the different experimental groups (the group of pure agarose was not evaluated because of the low number).

STATISTICS

Normality of the data was calculated by a Chi-square test, and diversity of variance was determined by an F-test. The statistical significance of differences between means was verified in two sample t-tests, assuming unequal variances, significance of different percentages by a Chi-square test requiring independent samples. Correlation coefficients were determined after combining results of different time points and related to confidence limits. In all tests $P < 0.05$ was considered.

ELECTRON MICROSCOPY

Fresh prepared samples were immediately fixed in buffered 1.25% glutaraldehyde/2% formaldehyde solution over night. After

FIG. 6. Chondrocyte agarose-filled defect in a medial condyle 12 months after transplantation; (a) immunohistochemical staining of type II collagen, survey (original magnification 25×), (b), (c) toluidine blue staining, details (original magnification 100×). The defect was filled completely with well reconstructed repair cartilage, but within the defect differences appeared between right and left half. At the right (c) the cartilage had a cleft surface, showing a change to fibrous splitting with simultaneous loss of type II collagen and of proteoglycans (more pronounced) and detachment from the residual cartilage. At the left (b) the repair cartilage developed a tidemark and was tightly connected to the original cartilage. Here columnar arrangement of chondrocytes was found in the deep zone.

FIG. 7. Chondrocyte agarose-filled defects in the patellar groove (a) 12 months after transplantation, toluidine blue staining, (b) 18 months after transplantation, immunohistochemical staining of type II collagen. Details (original magnification 100×) show the transition (arrow) between repair and normal cartilage. (a) The graft on the right formed a more regularly structured cartilage with only a thin superficial layer of connective tissue and represented a precursor of completely regenerated cartilage. (b) The reconstructed area (right) was still discernible by its appositions to the normal cartilage (left), a somewhat more narrow cartilage layer, small clusters of chondrocytes, and the slightly uneven surface.

FIG. 8. Chondrocyte agarose-filled defect in a patellar groove 18 months after transplantation; immunohistochemical staining of type II collagen; (a) survey (original magnification 25×), (b) detail (original magnification 100×). The repair cartilage was nearly identical with the normal cartilage in histological and immunohistochemical features. The interruption of the calcified cartilage indicated the original defect area (arrows). Chondrocytes of the deep zone formed some small clusters [Fig. 8(b)].

postfixation in 1% osmium tetroxide for 2 h the specimens were dehydrated in increasing concentrations of acetone and embedded in low-viscosity epoxy resin. The blocks of resin were cut with the aid of a Dupont diamond knife to provide ultra-thin sections, post-contrasted with uranyl acetate and lead citrate, and investigated in a Siemens Elmiskop 101 electron microscope.

Results

EVALUATION OF THE CULTURED CHONDROCYTES IN VITRO

Rabbit chondrocytes were cultured in agarose gel and developed in similar way as described by Aydelotte *et al*. [33] for bovine articular chondrocytes. About two days after inoculation they started to form alcian blue positive halos surrounding the cells. After 10–14 days they penetrated the agarose gel extensively (not shown). The percentage of viable cells remained nearly constant at about 90% for two weeks. The number of cells increased only slowly; at 10 days it had reached about 150% of inoculation.

VALUATION OF THE HEALING PROCESS OF THE LIVING ANIMAL

A functional evaluation of healing was not possible, since the artificial defects were too small to affect the mobility of the knee joint. The reason for limiting the size of the defects was that indirect effects resulting from impaired mobility were minimized. Thus, it is noteworthy that the injuries set in our study did not lead to functional impairment of the joints.

DEVELOPMENT OF DEFECTS WITHOUT CHONDROCYTE GRAFTS (CONTROL GROUP/PURE AGAROSE)

Macroscopic examination 6 months after surgery revealed that defects that had been left empty were not completely filled by substitutes [Fig. 1(a)]. The defects remained well visible for several months [Fig. 1(c)]. After 12 and 18 months, respectively, they were filled with a white tissue, exhibiting macroscopically a rough, fibrous surface [resembling Fig. 1(a), defect below].

The histological and immunohistochemical examination showed that control defects were typically filled with replacement tissue consisting of two layers: a stratum of type II collagen negative connective tissue at the surface and a layer containing chondrocyte-like cells near the bone [Fig. 2(a)]. The upper layer showed the typical appearance of a fibrous tissue layer; most of the cells were small or fibroblast like and irregularly arranged. The extracellular matrix of this replacement tissue was not, or only slightly, stained with toluidine blue, and no type II collagen was detectable by immunohistochemistry. The fibrous character of this zone was discernible by polarization microscopy. With prolonged time all repair tissues of the control group began to show clefts at the surface.

The deeper layer adjacent to the bone showed features of articular cartilage. The envelopes and surrounding extracellular matrix of the chondrocyte like cells reacted with the monoclonal antibody against type II collagen and with toluidine blue and safranin-O. Type II collagen and proteoglycans were completely missing in two, forming only a narrow or incomplete zone (Fig. 2) in 10, dominating in four of the 18 control specimens.

In three cases cartilaginous repair tissues were seen filling the original gap, but clearly separated from the host tissue; they never developed a tidemark and did not last more than 1 year. However, lateral clefts in the bone matrix that represented original margins of the defects persisted in 78% of control defects. Toluidine blue staining could fail in the normal cartilage adjacent to the defective area where type II collagen immunohistochemical staining was positive.

Defects sealed with pure agarose were not completely filled after 12 months, but macroscopically the grafts showed a cartilaginous appearance. The synovium did not show signs of inflammation. However, healing of the defect was less efficient after transplantation of pure agarose than in defects that were left empty [Fig. 3(a)]. Usually, the defects were poorly filled with fibrous replacement tissue formed by fibroblast like cells [Fig. 3(b)]. The repair tissue did not show metachromatic staining. Only the border of the defect was covered with a thin layer of cartilaginous tissue exhibiting weak staining by toluidine blue.

DEVELOPMENT OF DEFECTS CONTAINING TRANSPLANTED CHONDROCYTES

Macroscopically, most of the defects filled with chondrocytes embedded in agarose gel developed hyaline cartilage-like tissue at all periods examined [Figs. 1(b) and (d)]. The repair tissue had an appearance similar to the surrounding

cartilage, and was sometimes difficult to identify [Fig. 1(d)]. Few defects were similar to control. The patellar grooves and the femural heads were contoured normally. The synovium did not exhibit any signs of inflammation.

The histological and immunohistochemical results were in accordance with these findings, but revealed a high degree of variability. The repair tissue could be classified into three groups: (1) tissue similar to untreated defects, (2) tissues characterized by extensive cartilaginous areas in the deep zone and only a thin layer of type II collagen negative connective tissue at the surface, (3) tissue revealing almost perfect morphology of normal cartilage.

(1) In the first group the repair tissues showed a two-layered structure of a thin cartilaginous zone near the bone covered with type II collagen negative connective tissue (Fig. 4). Typically, 12 months after transplantation they still contained only a narrow type II collagen positive zone next to the bone [Fig. 4(a)]; sometimes as single type II collagen positive areas of insular shape in the depth [Fig. 4(b)]. The results of this group were considered negative, but in general they were not worse than the control group. In some cases a loss of the graft may have caused these results.

(2) In the second group the repair tissues contained pronounced cartilaginous, cell-rich areas (Figs 5 and 6). The arrangement of chondrocytes within the repair cartilage included different clusters of chondrocytes with diffuse cellular orientation, poorly resembling the characteristic spindle-shaped chondrocytes in healthy articular cartilage [Figs 5(b) and 6(b)]. The surface was intact or cleft (Fig. 6), but often the superficial repair tissue was converted into loose fibrous, type II collagen negative connective tissue [Fig. 6(c)]. Lateral merging with the adjacent cartilage varied from intimate connection of the matrices to open cracks. Defects with an imperfect transition between the graft and the adjacent cartilage mostly resulted in different surface levels and tended to be overgrown with fibrous material, and to degenerate by splitting the surface. Like in the controls, the calcified cartilage adjacent to the defect never merged with the grafts. Even after formation of a tidemark in the graft, no homogenous apposition in the area of calcified cartilage was observed.

(3) Best results were summarized in the last group (Figs 7 and 8). In this group the repair tissues revealed a smooth articular surface and were tightly connected to the adjacent cartilage. Only the transition of the calcified cartilage was interrupted. New subchondral bone was formed and the outline of the defect in the depth was dissolved. The cellular organization in the graft and the tidemark were nearly identical to that of the original articular cartilage. A superficial zone, radial zone, and a zone of calcified cartilage were recognized showing typical arrangements of single chondrocytes, columns and groups of chondrocytes, respectively. Also the pattern of proteoglycans and type II collagen characteristic for articular cartilage were detected by metachromatic staining and immunohistochemistry, respectively. Electron microscopic studies revealed, that the extracellular matrix in the repair tissue showed a similar appearance as in normal cartilage also at the supramolecular level [Fig. 9(a) and (b)]. Mature, typically cross-banded collagen fibrils were seen. They were dispersed in a loosely arranged pattern identical to that of natural cartilage fibers. In accordance with the fibrous appearance of the superficial zone of the repair tissue, bundles of parallel oriented fibrils were seen in electron microscopic images of this area [Fig. 9(c)]. Only residues of pure agarose were identified as less contrasted matrix areas without fibrillar structures [Fig. 9(d)].

EVALUATION BY HISTOLOGICAL AND MORPHOLOGICAL GRADING

For semiquantitative evaluation of the quality of the cartilage repair in chondrocyte transplants and control experiments, all specimens were graded according to seven histological and morphological criteria as listed in Table I. Although there was generally a high degree of variability, all chondrocyte implants recieved higher scores in all parameters in comparison to control explants. Concerning ‘proteoglycan staining’ and ‘type II collagen staining’, as well as ‘cellular arrangement’, an about 1.5-fold increase over controls was observed. Without exception, the best results were obtained in the 18 months chondrocyte transplant group (Fig. 10). A clear statistical improvement was achieved in the total evaluation and in the criterion ‘superficial zone’ at every period of time, and with progressing duration significant improvement was found for the criteria ‘type II collagen’, ‘cellular arrangement’, ‘tidemark’ and ‘lateral merging’.

In order to analyze whether positive effects correlate in several criteria, correlation coefficients were determined between any 2 parameters listed in Table I. In control

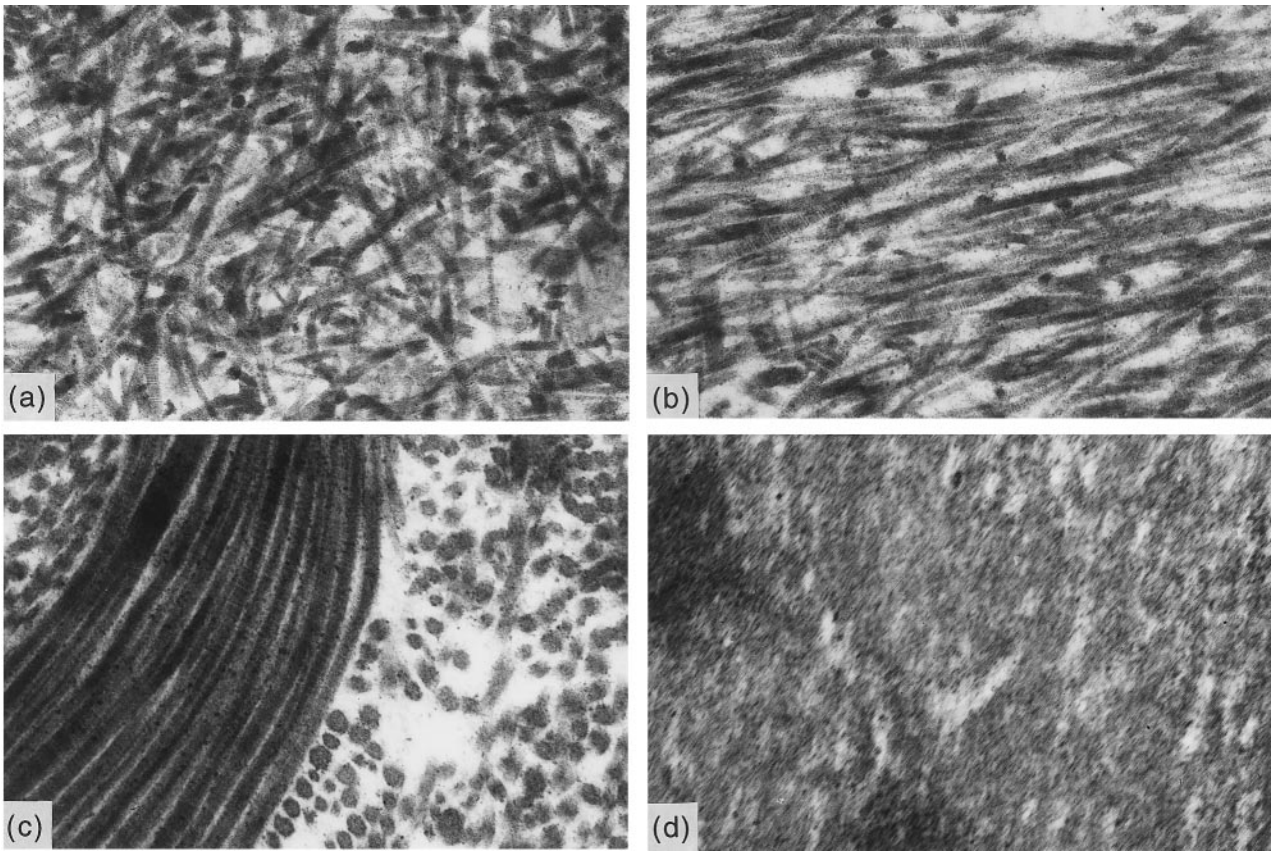

FIG. 9. Electron micrographs of the interterritorial matrix 12 months after transplantation of chondrocytes containing agarose (original magnification all 30 000×); (a) middle zone of the repair tissue, (b) superficial zone of the repair tissue, (c) middle zone of the adjacent normal articular cartilage: beside the bundle of fibrils on the left fibrils were oriented perpendicular to the image plane and were cut, (d) area without fibrils, indicating residues of agarose.

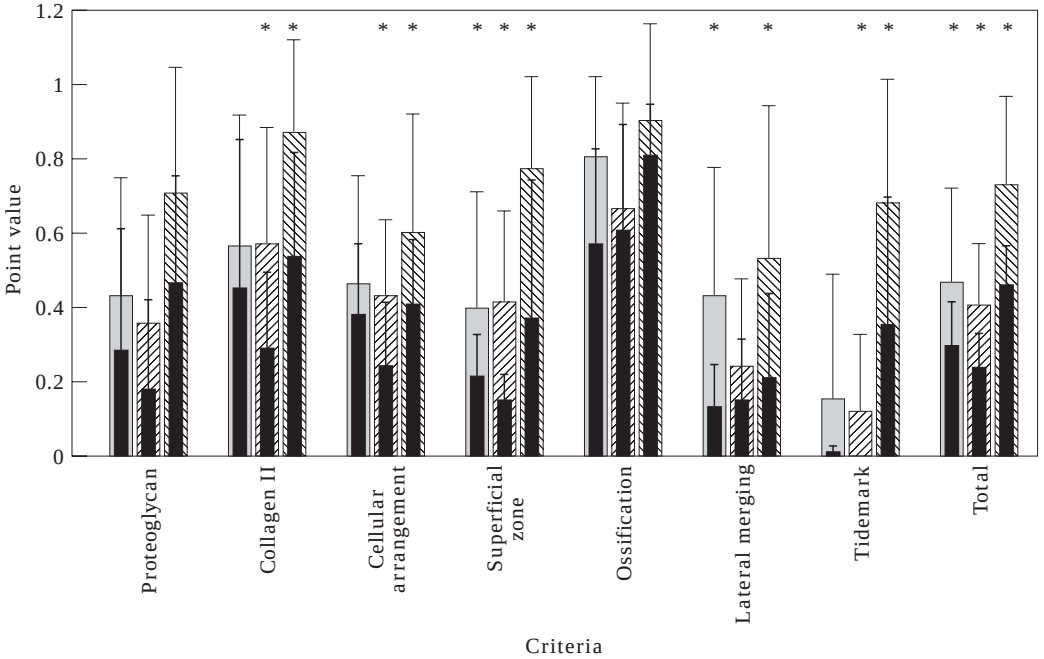

FIG. 10. Results of the evaluation of defects with the help of selected criteria. Three columns of one group in the background represent the total means of chondrocyte grafts ($N = 15$; 16) at 6 months (left), 12 months (middle) and 18 months (right); black columns in front represent the means of corresponding controls ($N = 6$). Significant differences between controls and grafts are marked by asterisks.

Table II

Correlation coefficients of the different possible pairs of criteria separated for controls (in the top righthand corner) and grafts (in the bottom lefthand corner, shaded). Values above the confidence limit (grafts 0.25, controls 0.4 $P < 0.05$) are marked by asterisks

Criterion	Proteoglycan	Type II collagen	Cellular arrangement	Lateral merging	Tidemark	Superficial zone	Ossification
		Controls	Controls	Controls	Controls	Controls	Controls
Proteoglycan	Grafts	0.83*	0.50*	0.08	–0.03	0.53*	0.22
Type II collagen	0.81*	Grafts	0.54*	–0.03	–0.19	0.50*	0.16
Cellular arrangement	0.71*	0.68*	Grafts	0.46*	0.19	0.33	–0.21
Lateral merging	0.73*	0.58*	0.73*	Grafts	0.24	0.29	–0.23
Tidemark	0.73*	0.59*	0.74*	0.63*	Grafts	–0.21	0.39
Superficial zone	0.65*	0.58*	0.45*	0.44*	0.62*	Grafts	–0.08
Ossification	0.42*	0.47*	0.42*	0.36*	0.44*	0.14	—

experiments as well as chondrocyte grafts a high correlation factor between 'proteoglycan' and 'collagen staining' was found ($r = 0.83$ and 0.81, respectively). In the transplantation group all pairs of criteria except one correlated well at a high correlation coefficient above the confidence limit (Table II).

The superior quality of the chondrocyte implant tissue as compared to control repair tissue is clearly depicted in Fig. 11, showing the percentage of specimens in each of four categories according to the criteria given in Table I. Each bar represents the mean value of the three time points for each group. By all criteria, a higher number of specimens of the cartilage implant group was graded in the categories 0.75 and 1.0 as compared with control specimens which were graded for the most part in the lowest (0.25) and second lowest (0.5) category (Fig. 11).

The extent of improved cartilage repair by the implanted chondrocytes over the controls becomes also apparent in another type of graphical summary of the histomorphological evaluation. In Fig. 12 the single scores of each criterion are added up, thus allowing a maximum of seven points for the best result. The bars indicate the percentage of specimens categorized under each score value. The figure demonstrates that (1) after 18 months both control specimens and chondrocyte grafts reached higher scores than in the 6 or 12 months samples, (2) chondrocyte graft samples of all time points were categorized at a high percentage in higher scores than controls, and (3) only the chondrocytes group contained samples (47% at 18 months) that were classified in the highest possible score of 6–7 points. After summing up results of all time points the differences between controls and grafts were statistically significant for the categories 1–2 points (decreased after grafting) and the category 6–7 points (increased after grafting) in the Chi-square test. However, this reflected an overall improvement, since also the percentage of specimens graded in the 0 to 3.5 point range (54% of chondrocyte grafts, 83% of controls) and in the 3.5 to 7 point range (46% of grafts, 17% of controls) differed significantly ($P < 0.05$). In the

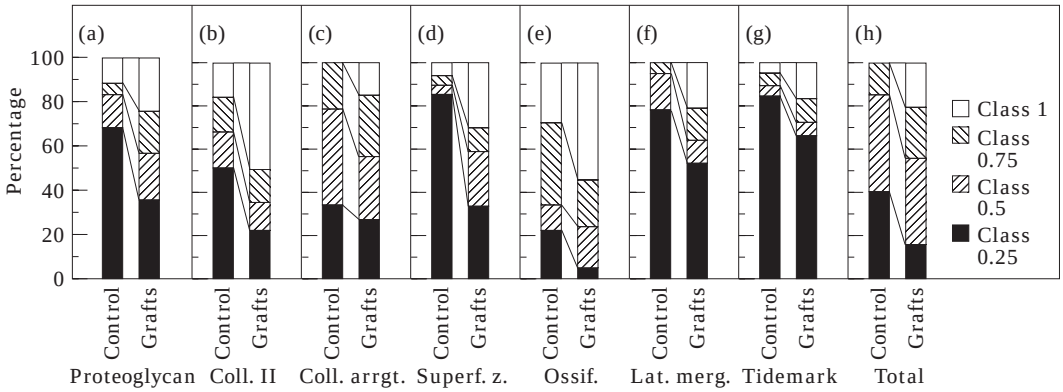

FIG. 11. Evaluation of the quality of the repair cartilage according to the parameters listed in Table I. The parts of the columns visualize the percentage of specimens, grouped into four classes 0–0.25 points, 0.25–0.5 points, etc.

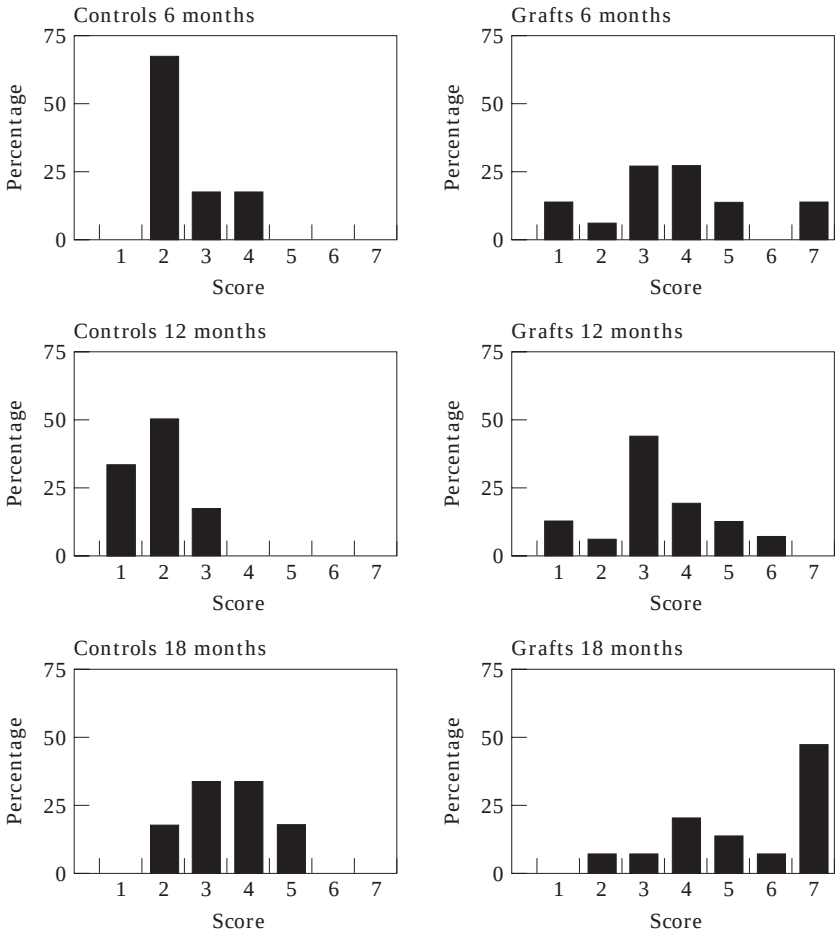

FIG. 12. Total scores of experimental and control specimens, taking into account all seven parameters. Bars reflect the percentage of specimens, categorized under each score value.

transplantation group 24% of the grafts (11/46) were evaluated with more than 5 points, whereas the best control achieved 4.45 points.

Discussion

The development of agarose systems for the cultivation of isolated mammalian chondrocytes started in the early seventies [34]. A series of studies has used this system since then and proved its potency to investigate phenotype, metabolism and differentiation of chondrocytes *in vitro* [e.g. 16, 33, 36]. It was shown that chondrocytes maintain their phenotype and synthesize and secrete an extracellular matrix containing proteoglycans and type II collagen when cultured in appropriate agarose gels immediately after enzymatic digestion [35, 36].

Also specific features of chondrocyte subpopulations have been demonstrated to be stable under these conditions [33]. Here we demonstrate that the transplantation of allograft chondrocytes embedded in agarose gel in full-thickness cartilage defects is a suitable method to repair articular cartilage defects in rabbits. The applied histological and immunohistochemical methods allowed both a reliable characterization of the repair tissue and insight into the reconstitution of the integrity of the defective areas.

ACCEPTANCE OF AGAROSE AND ALLOGRAFTS

There were no signs of graft-versus-host rejections or infiltration by immune cells, although immunological parameters including antibodies against agarose were not determined. Future studies will have to clarify the fate of agarose in chondrocyte agarose gels after transplantation. Hydrolytic degradation seems to be possible, since serum and tissue fluids contain a wide spectrum of glycosidases. Results from electron microscopic studies favour a rather complete replacement of the agarose matrix (unpublished). Similarly, also

in other transplantation studies with allograft chondrocytes [14, 25, 37], allografts of cartilage [7, 8] and periosteal allografts [38, cf. 9] rejection reactions were absent or low. This is of particular importance for allografts as it opens the possiblity to use material from a younger donor. Healing success depends to a large extent on the age of the donor. It has been shown that the healing potential of different kinds of grafts decreases with increasing age of the donor [cf. 38]. Robinson *et al*. [39] observed the formation of cartilaginous tissue in a chicken model, if embryonic chondrocytes were used, while even three-weeks old postnatal chondrocytes failed and formed a fibrous tissue. In the rabbit also chondrocytes from four-weeks old donors were reported to function [25]. In the study presented here chondrocytes from 1 to 2 weeks old rabbits were used; transplantation experiments with older donors are likely to be less successful.

EVALUATION CRITERIA

Seven criteria were chosen to evaluate specimen by a grading score which covered essential features of cartilage repair tissues. Although the criteria chosen may not appear of equal value, they turned out valuable for unbiased grading of the results when used in this combination. According to these criteria, the outcome of the chondrocyte implantation experiments ranged between unsuccessful repair to nearly perfect reconstitution of articular cartilage. Imperfect results of transplantations were in the same range as untreated defects. For this reason, the evaluation data of control and chondrocyte grafts underwent careful statistical analysis. This was important, since even control defects showed a wide variability.

SPONTANOUS HEALING OR CHONDROCYTETRANSPLANTATION?

Conflicting results about spontaneous healing of full-thickness defects in rabbits were published [1, 25, 40, 41], but there was agreement that the formation of hyaline like cartilage was only transient and was usually followed by conversion into a more fibrillated tissue. It was shown that at 50 days the repair cartilage was inferior to normal articular cartilage by histological criteria and histological findings and concerning the expression of type II collagen mRNA [41]. Our control results indicate that spontanous filling of cartilage defects may occur fast and probably irreversibly by replacement with fibrous tissue, but also by chondrocyte like cells and cartilaginous extracellular matrix. Differences among individual cases may result from morphological and constitutional variability between individual animals and on the depth of the subchondral lesion which is critical for the connection with the bone marrow and hence on the access to inflammatory- and bone marrow stem cells [3]. These are the major source of chondroprogenitor cells in spontaneous healing of cartilage defects [1]. Our data show that by transplantation of agarose-cultured chondrocytes significantly better results were obtained than in control defects. In nearly 50% of the cases the chondrocyte implants gave rise to a repair cartilage similar to hyaline articular cartilage with hyaline morphology and an extracellular matrix containing type II collagen and proteoglycans. In about 25% of cases even nearly perfect cartilage reconstruction was achieved.

INTEGRATION OF THE IMPLANTS

In several control and chondrocyte implant experiments postsurgical loss of the grafts was seen despite fixation of the grafted material with a fibrin glue. The mechanical stability of the agarose implants is low, and thus they may get lost during the repositioning of the knee joint. The approach of Hunziker and Rosenberg [4] to pretreat injured cartilage surfaces of partial thickness defects with chondroitinase or other enzymes to improve cellular adhesion will certainly contribute to the success of chondrocyte implantation. Early injuries of the surface of the gel may be the cause for insufficient quality of the superficial zone of the repair cartilage.

Critical for integration of the graft is the rapid formation of a tight contact between graft and surrounding tissue which requires infiltration of the fibrin glue and merging of the newly formed cartilage matrix with the surrounding matrix. Following this, the survival of the chondrocytes depends on their ability to further synthesize cartilage specific matrix components and to accumulate a pericellular matrix.

Perfect repair of the defect by hyaline cartilage also requires that the chondrocytes proliferate in a controlled manner by forming chondrones and differentiate according to their localization in the joint in order to build up specific cartilage zones adapted to the adjacent tissue. Decisive signals for site-specific differentiation have to be provided by the surrounding tissues. Agarose as implantation medium has the advantage of permitting almost free flux and diffusion of chemical signals, growth

factors or cytokines, and permits the formation of a new cartilage matrix.

Most of the chondrocyte grafts integrated with the adjacent cartilage above the tidemark and with the subchondral bone, but never with the calcified cartilage. Although the quality of the connections of the graft to the normal uncalcified cartilage were highly variable, undisturbed transitions between the graft and the adjacent cartilage were found. We consider this a remarkable advance over other similar methods [13, 25].

RECONSTITUTION OF SUBCHONDRAL BONE

In 89% of the grafts new subchondral bone formed in place of the original bone that was destroyed upon setting the drill hole. Bone trabecules developed in the deep zone of the graft, enclosing small residual chondrocyte islands. Mineralized cartilage islets indicating endochondral ossification were only rarely observed, but in all cases of perfect healing a zone of calcified cartilage formed in the deep zone that was separated by a tidemark from the upper cartilage zone. In control defects frequently ossification proceeded from the deep zone, leading to complete loss of cartilage when coinciding with destruction of the articular surface. Also after transplantation of chondrocytes ossification was protruding too far in 24% of the cases [compare Fig. 11(e)], but never as far in control defects, suggesting that the chondrocyte grafts produced signals which limited ossification.

However, low correlation to other parameters of restitution indicated that ossification proceeded as a relatively independent process.

QUALITY OF THE CARTILAGE IMPLANT IN LONG-TERM EXPERIMENTS

A crucial advantage of the chondrocyte implants over the control group was the fact that essential features of hyaline articular cartilage were preserved or even improved with prolonged time. Surface destruction became visible in the control group after 12 months, whereas in the group of transplanted chondrocytes intact surfaces maintained at least until 18 months. Moreover, the development of transplanted chondrocytes was a more ordered process than the intrinsic repair of control defects.

CHONDROCYTE-AGAROSE IMPLANTS IN COMPARISON WITH OTHER APPROACHES

In comparison to other approaches to restore cartilage defects, such as the procedure of Brittberg and coworkers [12, 13], who transplanted autologous chondrocytes expanded in monolayer culture under a periosteal flap, the histological and morphological quality of the chondrocyte implants presented here seems superior. Agarose may be more suitable for maintenance of the chondrocyte phenotype than carbon fibers or other fibrous material used for mechanical stabilization of chondrocyte implants [13]. Furthermore, there is ample evidence in the literature that three dimensional culture is superior to monolayer culture for stabilizing the chondrocyte phenotype [16, 42, 43]. In monolayer culture chondrocytes tend to transform into fibroblast-like cells and switch to type I collagen synthesis. Although from this state cells can return to the chondrocytic phenotype [16, 43], they also may develop to hypertrophic cells [44] or dedifferentiate irreversibly when cultured too long [16, 44]. On the other hand, data by Brittberg *et al*. [13] imply that coverage of cartilage defects with periosteum supports the development of hyaline cartilage also after transplantation of chondrocytes embedded in agarose by protecting the implant from mechanical injuries and from influences of interfering cells and molecules from the synovia like inflammatory mediators or degrading enzymes.

In conclusion, the study reported here confirms that the application of agarose as scaffold in chondrocyte transplantations clearly improved healing in comparison to defects without grafted chondrocytes. In the gross evaluation 46% of chondrocyte grafts ($N = 46$) developed good healing results (17% in controls, $N = 18$), 24% showed an extent of recovery and a quality of repair by hyaline cartilage never observed in controls. Evaluation of the specimens by seven histological/morphological criteria demonstrated a significant improvement of the quality of the repair tissue and a markedly lower extent of degeneration over time of the chondrocyte implants in comparison to controls. Thus, transplantation of chondrocytes embedded in agarose represents an alternative to other systems [12, 13, 25, 28], and produced results that are encouraging the further development of this method.

Further investigations have to clarify the influence of chondrogenic factors on the development of the transplant, the early processes in the first days and weeks, and will have to deal with the improvement of the surgical technique.

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